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EFFECTS OF TREE PHYTOCHEMISTRY ON THE INTERACTIONS AMONG ENDOPHLOEDIC FUNGI ASSOCIATED WITH THE SOUTHERN PINE BEETLE

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Abstract—We examined the interaction between host trees and fungi associated with a tree-killing bark beetle, *Dendroctonus frontalis*. We evaluated (1) the response of four *Pinus* species to fungal invasion and (2) the effects of plant secondary metabolites on primary growth of and secondary colonization of three consistent fungal associates. Two of these fungi, *Entomocorticium* sp. *A* and *Ophiostoma ranaculosum*, are obligate mutualists with *D. frontalis*, and the third associate is a blue-staining fungus, *O. minus*, that is commonly introduced by beetles and phoretic mites. *O. minus* negatively affects beetle larvae and in high abundance can impact *D. frontalis* population dynamics. Size of lesions formed and quantity of secondary metabolites produced in response to fungal inoculations varied significantly among *Pinus* species. However, monoterpene composition within infected tissue did not significantly vary across treatments. While all eight tested metabolites negatively affected the growth rate of *O. minus*, only 4-allylanisole, *p*-cymene, and terpinene reduced the growth of the mycangial fungi. Surprisingly, growth rates of mycangial fungi increased in the presence of several secondary metabolite volatiles. *O. minus* out-competed both mycangial fungi, but the presence of secondary metabolites altered the outcome slightly. *O. ranaculosum* out-performed *E.* sp. *A* in the presence of dominant conifer monoterpenes, such as α- and β-pinene. Volatiles from the mycangial fungi, particularly *E.* sp. *A*, had a negative effect on *O. minus* growth. In general, phloem phytochemistry of particular *Pinus* species appeared to alter the relative growth and competitiveness of mutualistic and non-mutualistic fungi associated with *D. frontalis*. The outcome of interactions

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among these fungi likely has important consequences for the population dynamics of *D. frontalis*.

Key Words—*Dendroctonus*, *Ophiostoma*, *Entomocorticium*, monoterpenes, plant defenses, competition, mycelial interactions, antagonism, symbiosis, resin.

INTRODUCTION

The oleoresin system of pines is a primary mechanism of tree resistance against subcortical invasion by insects and pathogens (Paine et al., 1997; Trapp and Croteau, 2001). The physical properties of resin, particularly the quantity of resin flow, are important in deterring and excluding attacking insects such as tree-killing bark beetles (Smith, 1966; Raffa and Berryman, 1982; Christiansen and Horntvedt, 1983; Paine and Stephen, 1987; Franceschi et al., 2000). Alternatively, chemical properties of oleoresin are likely more important in disrupting the activity of insects within trees by altering the nutritional quality of subcortical tissues (Smith, 1963; Coyne and Lott, 1976; Hodges et al., 1985). Differences in the chemical composition and physical properties of oleoresin among pine species are thought to be related to differences in resistance to insects and pathogens (Hodges et al., 1979; Tisdale et al., 2003).

More specifically, differences in the oleoresin chemistry among tree species may indirectly affect tree-killing bark beetles by altering the colonization success (Christiansen, 1985; Raffa and Smalley, 1988), growth rate (Bridges, 1987; Ross et al., 1992), and competitiveness of fungi associated with beetles (Raffa, 2001). Mutualistic fungi are thought to enhance the access of beetles to phloem tissues either by helping kill the tree (pathogenic; Solheim, 1992; Paine et al., 1997), neutralizing secondary metabolites within phloem (Paine, 1984), or providing phloem nutrients directly or indirectly to progeny (Barras, 1973; Beaver, 1989). Additionally, mutualistic fungi that are antagonistic to, or resist displacement by, non-mutualistic fungi could contribute to the successful development of beetle larvae (Goldhammer et al., 1990; Klepzig and Wilkens, 1997).

Variation in primary phytochemistry (e.g., N, P, carbohydrates) and secondary phytochemistry (e.g., terpenes, phenolics) likely leads to differences in the relative success and abundance of mutualistic and non-mutualistic fungi (Raffa, 2001), which would influence beetle fitness and subsequently the initiation and speed of beetle population growth (Showalter and Filip, 1993; Trapp and Croteau, 2001). The growth of and interactions between beetle-fungal associates on substrates varying in primary and secondary phytochemistry, however, have not been thoroughly studied.

Here, we focus on three questions dealing with the effects of tree metabolites on the growth and competitiveness of fungi associated with bark beetles: 1. Do trees vary in their response to various fungi associated with bark beetles? 2. Are

growth rates of the various fungal species differentially affected by plant secondary compounds within plant tissues? 3. Are intraguild interactions among fungi influenced by tree-secondary metabolites and beetle by-products? We selected the fungal community associated with the southern pine beetles, *Dendroctonus frontalis* Zimmerman (Coleoptera: Scolytidae), as a study system because (1) *D. frontalis* colonize a broad range of *Pinus* hosts, but have differential reproductive success across pine species and sites (Hodges et al., 1979; Veysey et al., 2003), (2) the reproductive success of *D. frontalis* depends on the presence of mutualistic fungi living in phloem (i.e., endophloedic) (Barras, 1973; Goldhammer et al., 1990), and (3) the presence of non-mutualistic fungi appears to affect larval development and influence broad patterns in *D. frontalis* population dynamics (Lombardero et al., 2003; Hofstetter et al., unpublished data).

We addressed the first question by quantifying the response of four *Pinus* species to artificial inoculations of three prominent fungal associates of *D. frontalis*. We hypothesized that localized tree response (lesion size and chemical composition) to fungal infection is influenced by (1) environmental conditions and (2) tree species. To address whether the response by *Pinus* to fungal infection is an effective defensive mechanism against both fungal invasion and beetle colonization, we quantified the effects of secondary metabolites of *Pinus* on fungal colonization and growth. We used a paired-confrontation assay to determine whether secondary metabolites alter the ability of fungi to resist or suppress invasion, or colonize substrate currently occupied by another fungus. We hypothesize that the presence of particular secondary metabolites alters the competitive interaction between fungi associated with *D. frontalis*.

METHODS AND MATERIALS

Study System. *Dendroctonus frontalis* is a destructive forest pest in the southeastern United States (Price and Doggett, 1978; Coulson and Witter, 1984). This beetle is efficient at finding and exploiting potential hosts (Payne et al., 1978; Nebeker et al., 1993) and can kill large numbers of otherwise healthy, vigorous pine species (Schowalter and Filip, 1993). Successful host colonization and subsequent reproduction of *D. frontalis* depend on the presence of mutualistic fungi, *Entomocorticium* sp. A. and/or *Ophiostoma ranaculosum* (formerly *Ceratocystiopsis ranaculosus*; Jacobs and Kirisits, 2003), that are carried from tree to tree in specialized glandular structures (mycangia) within the prothorax of female beetles (Francke-Grosmann, 1967; Barras and Perry, 1972). The two mycangial fungi are only found in the presence of *D. frontalis*, and the symbiosis is apparently a product of coevolution (Harrington, 1993). In the presence of mycangial fungi, adult beetles lay more eggs, which produce larvae that grow faster, survive better, and produce larger adults (Barras, 1973; Bridges, 1983; Bridges and Perry, 1985;

Goldhammer et al., 1990). However, the two species of mycangial fungi are not equivalent; larvae that feed on *E*. sp. A grow faster and become larger than those feeding on *O*. *ranaculosum* (Goldhammer et al., 1990).

A blue staining fungus, *Ophiostoma minus*, is commonly associated with *D*. *frontalis* (Barras, 1970; Bridges et al., 1985). In southern pine forests, *O*. *minus* is primarily an ectosymbiont of *D*. *frontalis* (Nelson et al., 1934), but it may also be carried by other insects associated with *D*. *frontalis* (Hofstetter, 2004). *O*. *minus* is detrimental to developing beetle larvae (Barras, 1970; Goldhammer et al., 1990; Lombardero et al., 2003), apparently because it displaces the mycangial fungi or disrupts the interactions between larvae and mycangial fungi within phloem tissue (Ross et al., 1992; Klepzig and Wilkens, 1997). In the presence of blue-stain, adults burrow away, while larvae produce atypical, winding galleries and usually die (Barras, 1970; Lombardero et al., 2003). This is unlike beetle species that carry other species of blue staining fungi within their mycangium, in an apparent mutualism (Paine and Hanlon, 1994; Six and Paine, 1998). High levels of *O*. *minus* can contribute to the decline of local beetle populations and the collapse of regional epidemics (Hofstetter, 2004).

Fungal Cultures. We isolated mycangial fungi (*E*. sp. A, *O*. *ranaculosum*) directly from the mycangia of female *D*. *frontals* (methods similar to Barras and Perry, 1972) collected from beetle infestations in the Homochitto National Forest, Mississippi, in June 1998. We isolated *O*. *minus* from the surface of *D*. *frontalis* beetles collected from *D*. *frontalis*-infested bark, also collected in Mississippi, June 1998. We cultured five strains of each fungus on malt extract (2%) agar (MEA) and incubated them at 25°C in darkness.

1. *Do Trees Vary in Their Response to Different Fungi Associated with D*. *frontalis?* We conducted two experiments to assess the response of healthy trees to fungal inoculations. In the first, we tested the hypothesis (1a) that the localized tree response (lesion size and chemical composition) to fungal infection is influenced by soil nutrient availability. In 1993, 45 loblolly pines, 25 years of age, located within an even-aged, thinned loblolly pine plantation in Camp Beauregard near Pineville, LA (31°22′ N, 92°22′ W), were selected for fertilization treatments. Half of the trees (randomly chosen) were fertilized with di-ammonium phosphate (150 kg/ha of phosphorus and 134 kg/ha of nitrogen) during each winter from 1993 to 1998. In August 1998, we inoculated 15 fertilized and 15 unfertilized *Pinus taeda* with the three fungal species. Points of inoculation were 1 m above the ground and separated horizontally around the tree by more than 10 cm. We removed 3 phloem and bark disks (1.22 cm diam) from each tree using a sterile cork borer, inoculated the tree with one of the three fungi, and replaced and sealed the outer bark with duct tape. We randomly selected one of the five strains of each fungus for each inoculation. The inoculums consisted of a 5 mm diam agar plug of culture. After 14 d, we removed the outer bark from the inoculation site, and measured the phloem lesion area (length, width, and total area). We evaluated

fungal growth rate using an ANOVA model (JMP 3.2.1, SAS Institute Inc. 1997) that included the parameters fertilization treatment, fungal species, fertilization-fungus interaction, and tree (nested within fertilization). The parameter fungus strain (within fungal species) was not included in the model because no appreciable difference was observed in fungal growth rate among fungal strains. To correct for heteroscedasticity, we log-transformed lesion size.

In the second experiment, we tested the hypothesis (1b) that localized tree response (lesion size and chemical composition) to fungal infection is influenced by tree species, fungal species, and wounding treatments. We tested four *Pinus* species commonly attacked by *D. frontalis*. In May 1999, we inoculated (as above) 15 trees of *P. echinata* (shortleaf), *P. palustris* (longleaf), and *P. taeda* (loblolly) within one mixed-species stand in the Oakmulgee Ranger District of the Talladega National Forest, Alabama, and 15 trees of *P. virginiana* in the Bankhead National Forest, Alabama. Each tree was inoculated at separate points with each of the three fungal species and chitosan (product of fungal/beetle cell wall; methods of Klepzig and Walkinshaw, 2003) and 2% MEA control. All trees were 30–40 yr of age and 20–30 cm diam trunk (d.b.h.). We also measured phloem thickness and resin flow (methods of Lombardero et al., 2000) just before the inoculations. After 21 d, we removed the outer bark and measured the lesion area. After measurement, we removed one half of each lesion for phytochemical analysis (stored at -80°C) and the other half (stored at -5°C) for fungal isolations. We analyzed total N and C using a Carlo-Erba C:N Analyzer. We analyzed monoterpene content by placing phloem samples (1.00 g) from each lesion into HPLC grade pentane with a *p*-cymene internal standard for 24 hr then analyzed the pentane samples using a 6890 GC equipped with a 5973 MS (Hewlett-Packard Corp., Palo Alto, CA) with an HP-5MS 30 m length $\times$ 250 um ID $\times$ 0.25 um thickness column. The temperature program was 60°C for 1 min- 6°C/min to 200°C, then 15°C/min- to 250°C. Flow rate was 0.9 ml/min, and the injector temperature was 200°C. We identified compounds by their mass spectra and matched the retention time with known standards.

We evaluated lesion size (total area) and lesion phytochemistry using an ANOVA model (JMP 3.2.1) that included *Pinus* spp, fungal spp, *Pinus*-fungus interaction, and tree (nested within *Pinus*). No appreciable difference in lesion size was observed within fungal strains and, thus, was not included in the model. To correct for heteroscedasticity, we log-transformed lesion areas and square root-transformed secondary metabolite concentrations. We computed Pearson product-moment correlations among lesion size, total secondary metabolites, and tree traits with a Bonferroni correction. Individual compounds were analyzed with a MANOVA (same model as previous ANOVA, but with each individual terpene as a dependent variable). Wilk's lambda and Roy's Maximum Root statistic were used to quantify main effects for tree species, inoculation treatment and their interaction.

2. Are Growth Rates of Fungi Differentially Affected by Primary and Secondary Metabolites Within Phloem? We conducted three experiments to assess the effects of volatiles from tree secondary metabolites and fungal metabolites on fungal growth. First, we determined whether growth among fungi varies in the absence of secondary metabolites. Second, we determined whether fungal growth rates change in the presence of particular tree-secondary metabolites. Third, we determined whether volatile byproducts from neighboring fungi influenced fungal growth.

We hypothesized (2a) that differences in primary phytochemistry of trees influences the growth rate of each fungal species. We assessed the growth rates of fungi on intact phloem tissue from which volatile secondary metabolites had been removed. In August 1998, we removed a phloem disk (10 cm diam) from each of 15 fertilized and 15 unfertilized *Pinus taeda* at the Camp Beauregard site. We discarded the outer bark and autoclaved the intact phloem for 20 min at 121°C to volatilize secondary metabolites and sterilize. We divided each phloem disk into thirds and placed them on a thin layer of water agar within a Y-shaped Petri dish (Fisher Scientific Inc.). We then placed on each section a disk of MEA (5 mm diam) that had been previously colonized by *O. minus*, *O. ranaculosum*, or *E.* sp. *A*. The sealed dishes were then incubated at 20°C in the dark. Beginning three d after the inoculation, and every two d thereafter, we traced the area of phloem occupied by hyphae on the lid of the dish. Once the phloem disk was completely colonized by fungi, we removed the lids and analyzed growth (both area captured and linear growth/day) with a planimeter. Prior to inoculation, we analyzed total nitrogen and carbon of each phloem sample using a Carlo-Erba C:N analyzer. We recorded annual tree growth using dendrometer bands affixed at 2 m above ground, and measured tree height growth with a hypsometer. Lombardero et al. (2000) previously reported effects of crown size, fertilization, and drought conditions on tree growth and resin production at this site. Linear growth and area captured by fungi on phloem disks were quantitatively similar within each fungal species. For simplicity and consistency with other experiments, we used linear growth in our statistical tests. We evaluated fungal growth rate using an ANOVA model (JMP 3.2.1, SAS Institute Inc., 1997) that included fertilization treatment, fungal species, fertilization-fungi interaction, and tree (nested within fertilization). To correct for heteroscedasticity, we log-transformed fungal growth rate. We evaluated correlations among fungal growth rates and tree traits (yearly growth, height, resin flow, phytochemistry).

In the second experiment, we tested the hypothesis (2b) that particular compounds commonly found within lesion tissue reduce the growth of each fungal species. To determine the effects of individual compounds on the growth of each fungus, we placed a 0.5 cm disk of MEA colonized with actively growing hyphae of one of the three species (*O. minus*, *O. ranaculosum*, or *E.* sp. A) onto the center of a 100 × 20 mm Petri dish of 2% MEA. We tested seven volatile compounds

commonly found in oleoresin of yellow pines (Pearl, 1975; Hodges et al., 1979): α-pinene, (S,−)β-pinene, (R,+)limonene, γ-terpinene, *p*-cymene, 4-allylanisole (estragole), and myrcene (Sigma-Aldrich, Inc.). Each compound (1.0 ml) was absorbed on a sterile filter paper (55 mm diam) and placed inside the Petri dish and sealed with parafilm; this created an atmosphere that was approximately saturated with the compound. We incubated each plate upside down at 25°C in darkness. Each treatment and a control (filter paper alone) were replicated 10 times for each fungus. We traced the outer edge of fungal growth on the outside of the dish every 2 d using a map tracer. We measured growth in four directions (0°, 90°, 180°, 270°) and averaged them to give a value for each time period. We ended the assay when the fungus reached the end of the Petri dish.

In the third experiment, we tested the hypothesis (2c) that volatile compounds released by neighboring fungi negatively affect fungal growth. We measured the growth rate of *O. minus* in the vicinity of one of four fungi associated with *D. frontalis*. We placed newly inoculated malt-agar plates of *O. minus* (face down) 1 cm above a 20-d old plate of actively growing *O. ranaculosum*, *E.* sp. A, *O. minus*, *Leptographium terebrantis*, or blank malt-extract agar control. We measured the growth of the inverted *O. minus* culture every two d until the plate was completely colonized (∼10 d). We replicated each fungal combination 25 times within separate 60 cm^2 plastic Petri dishes. We analyzed the effects of volatile compounds released from fungi on growth of *O. minus* at 6 d using ANOVA (following square root transformation) followed by Tukey-Kramer HSD.

3. Are Intraguild Interactions Mediated by Secondary Metabolites? We conducted an experiment to assess the effects of secondary metabolites on interactions between fungi. We hypothesized that the presence of tree secondary metabolites alters the ability of each fungal species to resist and suppress invasion or capture territories occupied by another fungus. We quantified area of resources captured by each fungi and observed the interaction between paired fungi using a paired confrontation assay (*O. minus vs. O. ranaculosum*; *O. minus vs. E.* sp. *A*; *O. ranaculosum vs. E.* sp. *A*) on 2% MEA media in the presence of a secondary metabolite. We added an eighth treatment, uric acid, which is a large component of beetle frass and common in larval chambers. We replicated each combination and media treatment 10 times. We aseptically removed disks (5 mm diam) of colonized malt extract agar from actively growing colonies of each of two fungal species and placed the inoculum onto opposite sides of the Petri plate (100 × 20 mm). We placed the fungal disks upside down within 2 cm from the edge of the plate. We placed a filter paper (55 mm diam), saturated with a compound (1.0 ml), inside the Petri dish, or added uric acid (concentrations of 1% or 0.5% by volume) directly to media prior to poring agar into plates. We incubated the plates upside down at 25°C in darkness. We traced the outer edge of fungal growth on the outside of the dish every 2 d. At the termination of the experiment, we measured the area occupied (cm^2/2 d) and the linear growth of each fungus using a digital planimeter

for each time increment. We compared the qualitative interactions between fungi following a key by Porter (1924).

RESULTS

1. Do Trees Vary in Their Response to Different Fungi Associated with D. frontalis? Hypothesis 1a: Localized response (lesion size and chemical composition) to fungal infection is influenced by environmental conditions.-Fertilization of *P. taeda* had no effect on lesion size among fungal species ($F_{2,43} = 0.89$, $P = 0.41$). However, *O. minus* consistently produced larger lesions (mean $\pm$ std: 94.9 ± 57.9 cm^2) than both *E.* sp. *A* (2.4 ± 2.6 cm^2) and *O. ranaculosum* (5.4 ± 4.9 cm^2). Lesion sizes from *O. minus*-inoculations were positively correlated with percent nitrogen in uncolonized phloem ($r = 0.70$), lesion sizes from *O. ranaculosum* were negatively correlated with percent carbon in phloem ($r = -0.63$), and lesion sizes from *E.* sp. *A* were positively correlated with tree growth rate (change in trunk circumference) ($r = 0.55$). All other tree measurements were not correlated with lesion size ($P > 0.05$). Only *O. minus* was successfully isolated (i.e., producing a living culture) from lesion tissues. No fungi were isolated from phloem beyond the lesion area.

Hypothesis 1b: Localized response (lesion size and chemical composition) to fungal infection is influenced by tree species, fungal species, and wounding treatments.–Lesion size in response to fungal inoculations varied little among tree species (Table 1), although *O. minus* produced somewhat larger lesions in *P. taeda* and *P. virginiana* than *P. echinata* and *P. palustris* (Figure 1). The interaction between inoculation treatment and *Pinus* species had no effect on lesion size (Table 1). The only significant correlations (across trees within species) between lesion size and % nitrogen, % carbon, resin flow, or tree diameter (Table 2) was a negative relation between % C in *P. virginiana* and the lesion size from *O. minus* ($r = -0.74$).

The total amount of secondary metabolites within lesions varied among pine species and depended upon the fungal species that was inoculated (Figure 1B, Table 1). In general, *P. echinata* had higher volumes of secondary metabolites per gram of phloem, and *P. palustris* had lower volume of metabolites than the other two tree species. Inoculations of *O. minus* did not result in significant increases in total metabolites in any of the tree species relative to the other inoculation treatments (*O. minus* vs. mean of other treatments; $P > 0.05$). Six compounds were detected in moderate amounts: (in general order of most to least abundant) α-pinene, β-pinene, 4-allylanisole, myrcene, limonene, and camphene. Ratios of the compounds varied significantly among tree species (Table 1, Figure 2). For instance, *P. echinata* and *P. virginiana* had significantly lower concentrations of β-pinene and 4-allylanisole than *P. taeda* and *P. palustris* across all inoculation treatments ($P < 0.05$). Also, limonene concentration in *P. virginiana* was high

TABLE 1. ANOVA TABLE FOR LESION SIZE, TOTAL MONOTERPENES AND MANOVA FOR EACH MONOTERPENE QUANTITY (g/g phloem) IN THE FOUR *Pinus* SPECIES FOR EACH INOCULATION TREATMENT

Source	df	lesion size	total Terp.	α-pinene	β-pinene	myrcene	limonene	4-allylanisole	camphene
Pinus spp	3	0.76	2.91*	3.62*	3.58*	4.48**	5.53**	17.4**	1.04
Inculation treatment	4	80.4**	5.54**	21.4**	16.7**	12.83**	3.11*	12.2**	14.4**
Pinus spp × Inoculation	12	0.90	1.51	0.84	1.05	0.61	0.81	1.32	0.62
Tree {*Pinus* spp}	16	1.65	1.25	2.51**	2.79**	3.11*	6.27*	3.89**	1.53

Note. Wilks' lambda from MANOVA: *Pinus* spp: 0.092**, Treatment: 0.338**, *Pinus* × Treatment: 0.209**, Tree {*Pinus* spp}: 0.018**. *Roy's Maximum Root from* MANOVA: *Pinus* spp. 2.55**, Treatment: 1.62**, *Pinus* × Treatment: 1.44**, Tree {*Pinus* spp}: 2.78**
MSEs, from left to right (with $df = 94$): 0.707,11.19, 247.5, 77.34, 0.322, 3.86, 4.62, 0.039

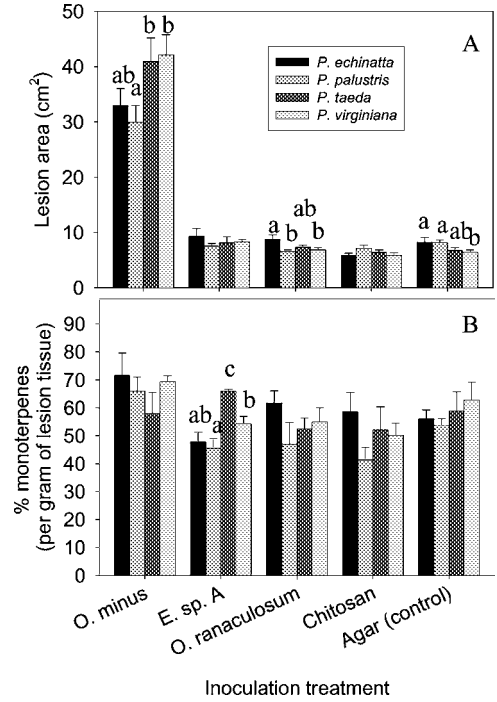

FIG. 1. (A) Lesion size (cm^2) and (B) monoterpene levels (including 4-allylanisole) in 4 *Pinus* species after 14 days in response to inoculation of *Ophiostoma minus*, *Entomocorticium* sp. *A*, *O. ranaculosus*, chitosan, and agar control.

relative to the other three *Pinus* species. Monoterpenes of low concentration (i.e., myrcene, limonene, and camphene) were not detected in some of the inoculation treatments within *Pinus* species (particularly inoculations other than *O. minus* in *P. echinata* and *P. palustris*).

2. Are Growth Rates of Fungi Differentially Affected by Plant Phytochemistry? Hypothesis 2a: Primary phytochemistry influences the growth rate of each fungus. Mean fungal growth on isolated phloem tissue varied across fungal species ($F_{2,43} = 291.1$, $P < 0.001$) with *O. minus* growing about twice as fast as *E.* sp. *A* or *O. ranaculosum* (mean $\pm$ SE $= 2.24 \pm 0.08$, 1.12 ± 0.03, and 0.97 ± 0.03 mm/d, respectively). Fertilization of *P. taeda* had no effect on fungal growth rates (for each fungus: $F_{1,14} < 3.1$, $P > 0.11$), nor did it interact with fungal species ($F = 0.52$, $P = 0.60$). Nitrogen content of phloem was similar between fertilized and unfertilized trees ($0.46 + 0.08$ vs. $0.43 + 0.08$; $F_{1,13} = 0.68$, $P = 0.42$). The growth of *O. minus* was negatively correlated with percent nitrogen in phloem and positively correlated with tree radial growth rate, but growth

TABLE 2. MEASURED TRAITS IN FOUR *Pinus* SPECIES (MEANS ± SD, $N = 15$)

	P. taeda[a]	*P. taeda*[b]	*P. echinata*[b]	*P. palustris*[b]	*P. virginian*[b]
Phloem % nitrogen	0.45 ± 0.08	0.21 ± 0.03	0.22 ± 0.03	0.21 ± 0.04	0.25 ± 0.02
Phloem % carbon	40.7 ± 4.7	50.3 ± 0.7	51.0 ± 1.8	49.0 ± 0.5	46.7 ± 1.1
Trunk diameter (cm)	24 ± 1	30 ± 4	27 ± 3	33 ± 4	28 ± 4
Constitutive resin (g/d)	0.7 ± 0.6	1.4 ± 0.7	1.8 ± 1.8	2.1 ± 1.8	0.2 ± 0.2
Tree height (m)	19.7 ± 1.2				
Tree growth rate[c]	8.9 ± 2.1				

[a] Located at Camp Beauregard, LA in 1998. N and C samples from Camp Beauregard were analyzed separately from Talladega samples.
[b] Located in Talladega National Forest, AL in 1999.
[c] Annual changes in tree circumference (cm) at breast height.

rates of mycangial fungi were not correlated with any measured traits of trees (Table 2). Average fungal growth rates on intact phloem were 17, 24, and 50% less than on 2% malt-extract agar (*O. ranaculosum*, *O. minus*, and *E.* sp A, respectively; $P < 0.01$ for all).

Hypothesis 2b: Volatile compounds commonly found within lesion tissue reduce the growth of fungal species. Fungal growth rates on malt-extract agar were highly sensitive to volatiles of tree-secondary metabolites (Figure 3). Growth of *O. minus* was significantly reduced in the presence of all compounds tested. *Entomocorticium* sp. *A* grew faster in the presence of myrcene, but grew slower in the presence of all other compounds compared to 2% malt agar alone. *Ophiostoma ranaculosum* grew faster in the presence of limonene, myrcene, and α- and β-pinene than on malt agar alone. All fungi grew slowest in the presence of volatiles from 4-allylanisole followed by *p*-cymene.

Hypothesis 2c: Volatile compounds produced by neighboring fungi reduce fungal growth. Growth rate of *O. minus* was generally reduced by the volatiles of other actively growing fungi (Figure 4). Aerial contact with actively growing cultures of *O. minus*, *E.* sp. *A*, and *O. ranaculosum* all reduced the growth of *O. minus* relative to the control; in the middle of the trial, when growth rates were most rapid, these effects were all significant ($P < 0.05$). Mycangial fungi (particularly *E.* sp. *A*) had the greatest negative affect on *O. minus* growth. Only *L. terebrantis* did not reduce *O. minus* growth rate relative to the blank control.

3. Are Intraguild Interactions Mediated by Secondary Metabolites? Hypothesis 3: The presence of tree secondary metabolites alters the ability of each fungal species to resist and suppress invasion or capture territories occupied by another fungus. The ability of fungi to resist or suppress growth by neighboring fungi was altered by the presence of particular secondary metabolites (Table 3). For instance, interactions between *O. minus* and *O. ranaculosum* were altered in the presence of limonene and high uric acid concentrations. All volatiles except *p*-cymene and α-pinene altered the interaction between *O. minus* and *E.* sp. *A* relative to

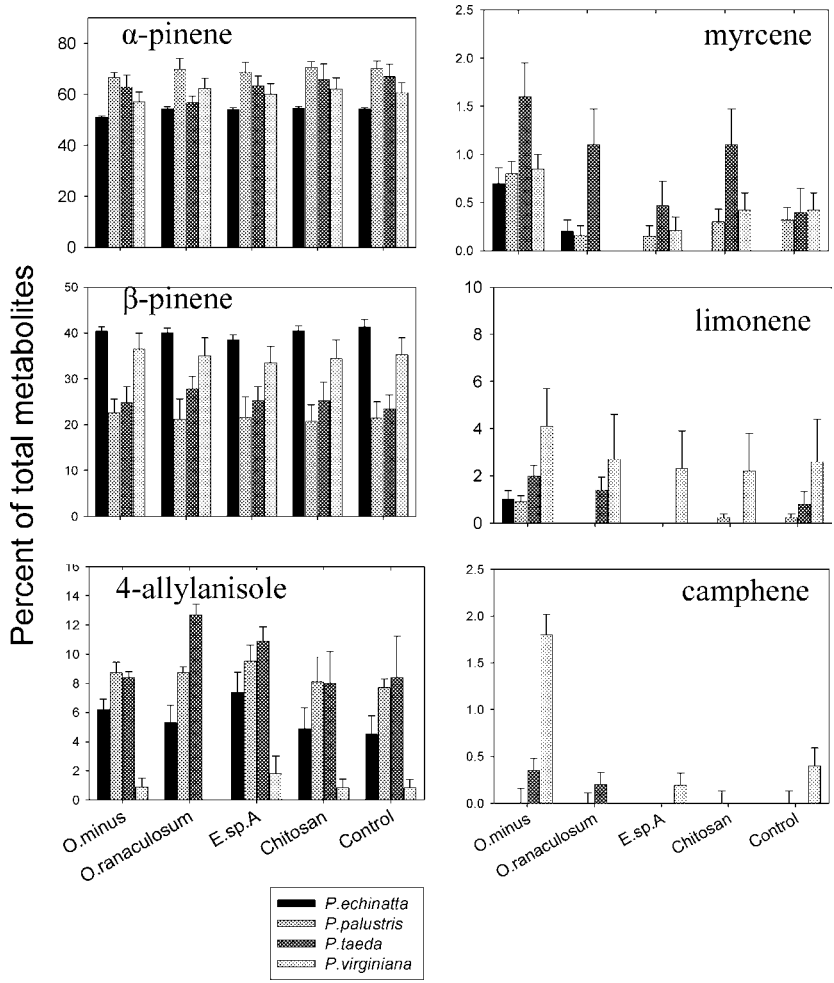

FIG. 2. Secondary metabolite concentrations in lesion tissues within *P. taeda*, *P. echinata*, *P. palustris*, and *P. virginiana* in response to an agar control, chitosan, *Entomocorticium* sp. *A*, *O. ranaculosum*, or *O. minus*.

the interaction observed between the two fungi on malt-extract agar alone. *Entomocorticium* sp. *A* and *O. minus* showed no signs of competitive interactions (no altered or slowed growth) in the presence of β-pinene. In the absence of secondary metabolites, *O. ranaculosum* captured about 46% of the area in competition with *E.* sp. *A* (control in Figure 5). However, *O. ranaculosum* captured significantly more resources in the presence of α-pinene, β-pinene, and 4-allylanisole, and

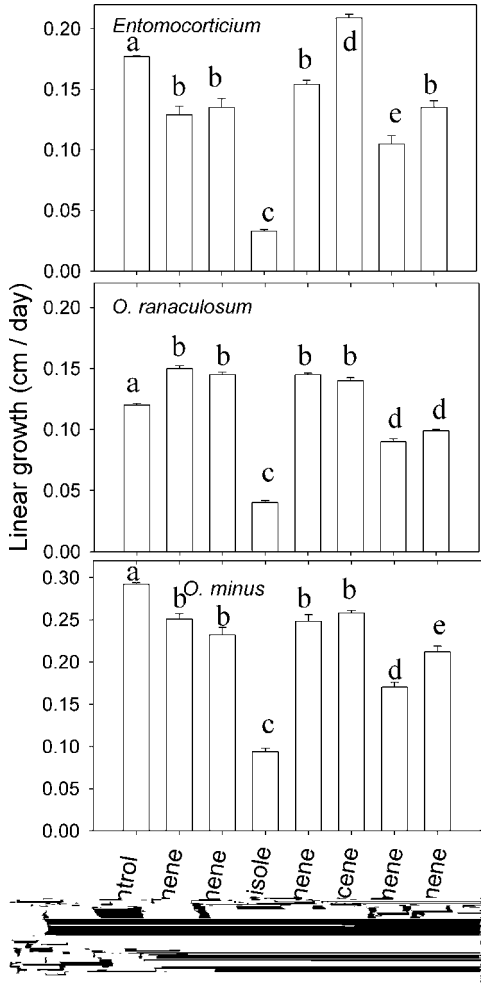

FIG. 3. Mean linear growth rate (cm/day) of each fungus on 2% malt-extract agar in the absence (control) or presence of volatiles from a particular monoterpene. Error bars indicate standard deviation and letters indicate significant differences across treatments ($P < 0.05$).

significantly less in the presence of myrcene, *p*-cymene and terpinene (Figure 5). *O. minus* always captured more area than either mycangial fungi (60–85% of the area; Figure 6). The amount of area captured by *O. minus* vs. mycangial fungi was highest in the presence of 4-allylanisole and similar in the other treatments. In the absence of secondary metabolites, but in the presence of *O. minus*, *E.* sp. *A* colonized more phloem area than *O. ranaculosum* (control in Figure 6).

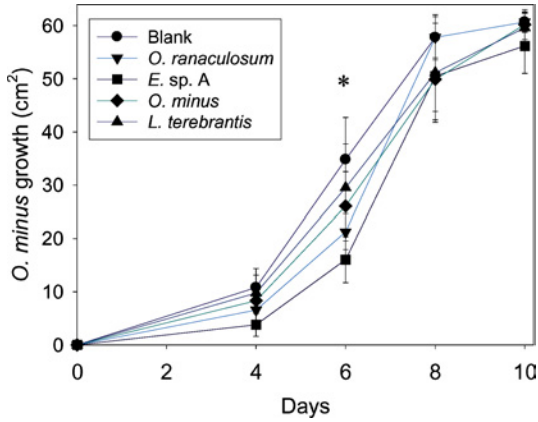

FIG. 4. Area growth (cm^2) of *O. minus* per day in presence of volatiles from other fungi associated with *D. frontalis*-infested trees, including other strains of *O. minus*. *Leptographium terebrantis* is usually found in the roots and does not usually colonize tissues inhabited by *O. minus*.

However, the relative performance of the two mycangial fungi (while in competition with *O. minus*) was altered by secondary metabolites; e.g., *O. ranaculosum* outperformed *E.* sp. *A* in assays with *O. minus* in the presence of α- or β-pinene volatiles (Figure 6).

DISCUSSION

1. Do Trees Vary in Their Response to Different Fungi Associated with D. frontalis? Secondary metabolites within lesion tissue vary depending on the infecting agent and pine species (Figure 2, Table 1). However, no clear pattern between chemical content and lesion size in phloem could be detected (Figure 1). Tree growth rate, d.b.h., % nitrogen or carbohydrates, and resin flow were correlated with lesion size in several tree species but there was no consistent pattern across all tree species. However, given that lesion size was not correlated with fungal growth rate on removed intact phloem from the same *P. taeda*, secondary metabolites (removed from intact phloem by autoclave) likely affect fungal growth ($\approx$lesion size) within trees. Greater induced response to *O. minus* relative to the other mycangial fungi *indicates* that (1) these tree species are highly sensitive (production of an elicitor within the tree; Delorme and Lieutier, 1990) to *O. minus* infection, (2) *O. minus* is pathogenic and thus triggers a greater response compared to that of the mycangial fungi, or (3) *O. minus* is capable of growing faster within resinous lesion tissues than the mycangial fungi. Particular secondary metabolites

TABLE 3. QUALITATIVE RESULTS FROM COMPETITION WITHIN PAIRED COMBINATIONS OF *E*. sp. A, *O. ranaculosum*, AND *O. minus* IN THE PRESENCE OF A MONOTERPENE VOLATILE OR URIC ACID ADDED TO GROWTH SUBSTRATE

Treatment	Pair combination		
	Entomocorticium vs. O. ranaculosum	*O. minus vs. O. ranaculosum*	*O. minus vs. Entomocorticium*
Control	1	2	3
p-Cymene	1	2	3
4-allylanisole	1	2	2,3
Myrcene	1,2[b]	2	2,3
Limonene	1[c]	1,2	2,3
α-Pinene	1,2[a]	2	3
β-Pinene	1	2	1,2
Terpinene	2[b]	2	2,3
0.5% Uric acid	1	2	2,3
1% Uric acid	1	2,3	2,3

Note. Following Porter (1924): "1" designates mutually intermingling growth; "2"-one fungus dominates, the other fungus shows little growth into other fungus; "3"-mutual slight inhibition (slowed growth) of both fungi than overgrowth by one of the fungi. Unless stated otherwise, *O. minus* dominated (grew over) the other fungus while the other fungus stopped growing. No two fungi where mutually inhibited at a distance greater than 2 mm. Two numbers per cell (e.g., 2, 3) indicate that replicates varied within the treatment.

[a] *O. ranaculosum* grew into area previously captured by *E*. sp. A.

[b] *E*. sp. *A* grew into area previously captured by *O. ranaculosum*.

[c] When fungi interacted, *E*. sp. *A* grew under *O. ranaculosum* (which grew only on the surface).

within induced resins may inhibit or slow growth of beetle-mutualistic fungi, or a reduction in available nutrients within lesion tissues might have a greater negative impact on beetle-mutualistic fungi than *O. minus*.

2. Are Growth Rates of Fungi Differentially Affected by Secondary Metabolites? Volatiles of secondary metabolites differentially affected the growth of fungus cultures (Figure 3). In the case of *O. minus*, all secondary metabolites reduced growth. Even with reduced growth rates, *O. minus* still grew faster than the mycangial fungi. Surprisingly, several secondary metabolites increased growth rates of the mycangial fungi relative to the growth rates in the absence of metabolites. Enhanced growth in the presence of a secondary metabolite might result from stimulated (increased) directional growth toward or away from the compound source (Rice, 1970), oxygenation of the compound (Knobloch et al., 1989), a specific response of the fungus to the optical rotation (chirality), number of double bonds (Cobb et al., 1968), or use as a carbon source (Paine and Hanlon, 1994). The two mycangial fungi responded differently to each compound, suggesting that each mycangial fungi might perform differently within different host trees. The major volatile constituents of induced phloem (and oleoresin) might not always enhance

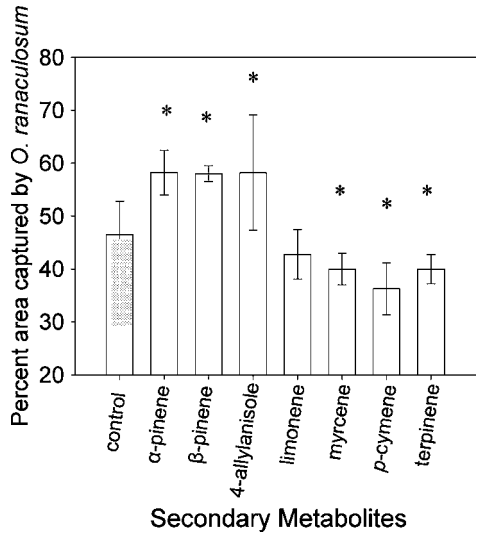

FIG. 5. Percent area captured after 30 days by *O. ranaculosum* compared to *E.* sp. *A* in the absence (control) or presence of a monoterpene (volatiles) in a paired-confrontation assay. Error bars indicate standard deviations and asterisks represent significant difference from control ($^{*}P < 0.05$, $^{**}P < 0.01$).

the resistance of trees to beetle invasion. Each fungus may also respond differently to various concentrations of each metabolite (Paine and Hanlon, 1994). However, small concentrations of secondary metabolites, like 4-allylanisole and terpinene can have strong negative effects on the mycangial fungi (Figure 3) and reduce the attraction of *D. frontalis* to host trees (Strom et al., 1994).

3. Are Intraguild Interactions Mediated by Secondary Metabolites? The relative growth and ability of the mycangial fungi *O. ranaculosum* and *E.* sp *A* to capture resources in the presence of *O. minus* was altered by secondary metabolites (Figure 6, Table 2). Thus, differences in compound concentrations or lack of particular compounds (e.g., no 4-allylanisole observed in *P. virginiana*) and the abundance of *O. minus* within trees may influence the relative frequencies of mycangial fungi within beetle infestations. In our competition studies, *E.* sp. *A* was more effective at reducing *O. minus* growth into colonized areas on artificial media than *O. ranaculosum* (Figure 6; Klepzig and Wilkens, 1997). However, secondary metabolites altered the relative ability of each mycangial fungus to compete with *O. minus*. For instance, *O. ranaculosum* was a relatively better competitor with *O. minus* than *E.* sp. *A* in the presence of α- or β-pinene. Goldhammer et al. (1989) found that high levels of uric acid in growth media reduced *O. ranaculosum* and *O. minus* growth, but increased the growth rate of *E.* sp. *A*. Uric acid

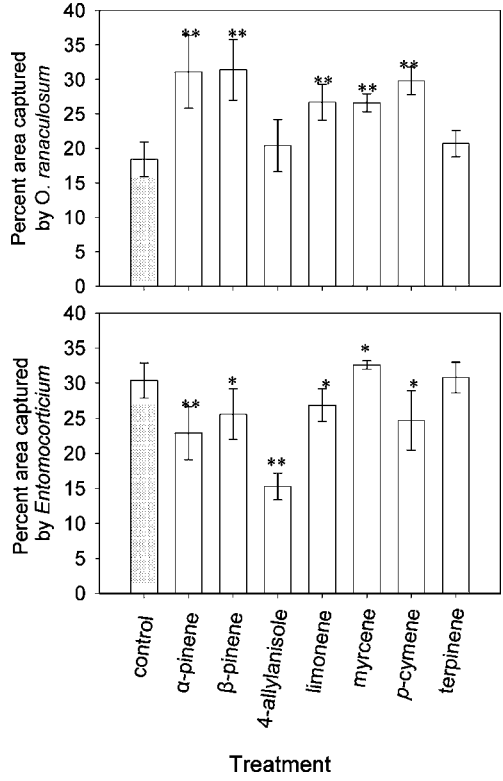

FIG. 6. Percent area captured after 30 days by *O. ranaculosum* or *E.* sp. *A* in paired assay with *O. minus* in the absence (control) or presence of a secondary metabolite (volatile). Error bars indicate standard deviations. Asterisks indicate significant difference from control ($^{*}P < 0.05$; $^{**}P < 0.01$).

also reduced *O. minus*' ability to capture resources previously captured by *E.* sp *A*. Uric acid levels likely increase in larval chambers as beetles develop, and, thus, become more important for competing fungi during the late larval or pupal stage. Phloem chemistry could affect the nature of interactions between fungi by altering the production, diffusion, or volatilization of fungal-produced compounds (Boddy, 2000).

Tree chemistry and interactions among fungi may select for the presence of multiple mutualistic fungi in beetle species that associate with mycangial fungi. Sampling of *D. frontalis* populations, ranging from one colonized tree to greater than 100 trees, have revealed the continual presence of both mycangial fungus (Bridges, 1983; Coppedge et al., 1995; Hofstetter, 2004). In fact, most bark beetle

species that have mycangial fungi appear to have two co-existing species of fungal mutualists (Six, 2002). Alternatively, the presence of mites (e.g., *Tarsonemus* guild) may promote the presence of a second mycangial fungus (e.g., "exploiter" species, Stanton, 2003) (Hofstetter et al., unpublished data).

Effect of Tree-Fungal Interactions on Beetle Populations

Differences in tolerance (represented by growth rate or competition) of the fungi to various compounds have ecological and economic significance. *O. minus* reduces survival of *D. frontalis* larvae (Lombardero et al., 2003) and reduces the growth rate and longevity of *D. frontalis* infestations (Hofstetter et al. unpublished data). Increased levels of *O. minus* within trees also reduce the economic value of wood as a result of blue staining in the xylem (Kreber and Byrne, 1994). All monoterpenes reduced the growth of *O. minus* and, thus, provide some defense against *O. minus* infection. High production of 4-allylanisole or terpinene, in particular, may reduce the relative abundance of blue-stain coverage within trees. Interestingly, increased growth and competitiveness of *O. minus* may minimize the destructive impacts of *D. frontalis* in pine plantations and forests by reducing beetle abundance (Lombardero et al., 2003; Hofstetter, 2004).

Differences in the frequencies of the two-mycangial fungi may affect beetle size, development rate, and reproduction within beetle populations (Goldhammer et al., 1990; Coppedge et al., 1995) and likely influences beetle population growth rate (Bridges, 1983; Ayres et al., 2000). Our results suggest that pine species composition within forests could affect the frequencies of each fungus within an infestation. For example, high levels of particular secondary metabolites within a stand of pines (e.g., shortleaf vs. longleaf) may favor the growth of *O. ranaculosum* relative to *E.* sp. *A*. Seasonal variation in secondary metabolites could generate seasonal variation in the relative abundance of mycangial fungi.

The frequency of each mycangial fungus within local beetle populations may change as beetle populations transition from low to outbreak densities. In endemic (low density) populations, *D. frontalis* usually colonizes trees that already have reduced defenses (e.g., lightning struck tree) that may favor the growth of *O. minus* and *E.* sp *A* relative to *O. ranaculosum* (Figures 3–5). Surveys of endemic populations (between September–December) by Bridges (1983) revealed that *E.* sp. *A* was more abundant than *O. ranaculosum* in 7 out of 8 *D. frontalis* populations. Alternatively, *O. ranaculosum* may become more prevalent in epidemic populations as a result of increased colonization of healthy (resinous) trees. Increased attack rates on trees may also favor *O. minus* growth (relative to mycangial fungi) if high attack rates result in lower secondary metabolites within host trees. Surveys of ten infestations during an epidemic in Alabama revealed that the relative proportions of *E.* sp. *A* and *O. ranaculosum* cycle throughout the year. Temporal variation in mycangial fungi may result from seasonal changes in beetle

attack rate, fungal growth rate, or interspecific interactions (Hofstetter, 2004). In any case, the relative frequency of each fungus in combination with the tree-host selection behavior of *D. frontalis* likely influences local population dynamics of *D. frontalis*.

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